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Signed M.W. Russell

Dated 8th June, 1992.

PRODUCTION OF ANTIBODIES

This invention concerns the production of human antibodies by recombinant DNA technology, and the use of such antibodies in the therapeutic and prophylactic treatment of human beings.

Antibodies, or immunoglobulins, are proteinaceous bifunctional molecules. One region, which is highly variable between different antibodies, is responsible for binding to an antigen, for example, the many different infectious agents that the body may encounter, whilst the second, constant region is responsible for binding to the Fc receptors of cells and also activates complement, a complex system of proteins responsible for cell lysis. In this way, antibodies represent a vital component of the immune response of mammals in destroying foreign microorganisms and viruses.

Antibodies are divided into different classes on the basis of the structure of the constant region. In humans for example, five major structural classes can be identified immunoglobulin G or IgG, IgM, IgA, IgD and IgE. Each class is distinguished on the basis of its physical and biological characteristics which relate to the function of the immunoglobulin in the immune system. IgGs can be further divided into four subclasses: IgG1, IgG2, IgG3 and IgG4, based on differences in the heavy chain amino acid composition and in disulphide bridging (see below for explanation), giving rise to differences in biological behaviour. A description of the classes and subclasses is set out in "Essential Immunology" by Ivan Roitt, Blackwell Scientific Publications.

An antibody molecule is composed of two light chains and two heavy chains that are held together by interchain disulphide bonds. Each light chain is linked to a heavy chain by disulphide bonds and the two heavy chains are linked to each other by disulphide bonds. Each heavy chain has at one end a variable domain followed by a number of constant domains, and each light chain has a variable domain at one

end and a constant domain at the other end. The light chain variable domain is aligned with the variable domain of the heavy chain. The light chain constant domain is aligned with the first constant domain of the heavy chain. The remaining constant domains of the heavy chains are aligned with each other. The constant domains in the light and heavy chains are not involved directly in binding the antibody to the antigen.

The variable domains of each pair of light and heavy chains form the antigen binding site. They have the same general structure with each domain comprising a framework of four regions, whose sequences are relatively conserved, connected by three complementarity determining regions (CDRs). The four framework regions largely adopt a beta-sheet conformation and the CDRs form loops connecting, and in some cases comprising part of, the beta-sheet structure. The CDRs are held in close proximity by the framework regions and, with the CDRs from the other domain, contribute to the formation of the antigen binding site.

The antibody chains are encoded by genes at three separate loci on different chromosomes. One locus encodes the heavy chain isotypes and there are separate loci for the κ and λ light chains, although a B-lymphocyte only transcribes from one of these light chain loci. The genes which encode antibody variable domains are generated during B-lymphocyte ontogeny by a process of recombination involving the joining of the V,D and J gene regions. A single B-lymphocyte will only use one heavy chain and one light chain recombined variable domain to ensure that it only has one antigen specificity. So only one allele of the heavy chain and one allele of the light chain are expressed by a single B-lymphocyte. This is known as allelic exclusion.

The exposure of an animal to an antigen by infection or immunisation, results in the production of different antibodies with different specificities and affinities. An antiserum obtained from the immunised animal will, therefore, be heterogeneous and contain a pool

of antibodies produced by many different lymphocyte clones. Antibodies thus obtained are referred to as polyclonal antibodies and this polyclonal nature has been a major drawback in the use of antibodies in therapeutic applications and in diagnostic assays.

A major step forward occurred in 1975 when Kohler and Milstein (Nature, 1975, 256, 495-497) reported the successful fusion of spleen cells from mice immunized with an antigen with cells of a murine myeloma line. The resulting hybrid cells, termed hybridomas, have the properties of antibody production derived from spleen cells and of continuous growth derived from the myeloma cells. Each hybridoma synthesizes and secretes a single antibody to a particular determinant of the original antigen. To ensure that all cells in a culture are identical, i.e. that they contain the genetic information required for the synthesis of a unique antibody species, the hybridomas resulting from cell fusion are cloned and subcloned. In this way, the cloned hybridomas produce homogeneous antibodies of the original animal species from which the spleen cells were derived.

The ability to produce monoclonal antibodies has revolutionised the diagnosis of many diseases and provides the possibility of prevention and immunotherapy of numerous pathological disorders. Unfortunately, foreign antibodies namely antibodies of non-human species, such as a mouse or a rat, administered repeatedly to a human for vaccination or treatment, will be recognised by the individual's immune system and are likely to cause an undesirable anti-globulin response. This anti-antibody response is due to the foreign origin of the constant domains and the four framework regions. The result of this response is likely to be the neutralising of the therapeutic antibody, and the triggering of harmful anaphylactic or allergic reactions. Furthermore, non-human monoclonal antibodies do not fix human complement particularly well and are less likely to trigger non-specific mechanisms of cell clearance such as antibody - dependent cell - mediated cytotoxcity (ADCC), in view of the differences in effector function of the constant region of the antibody. Non-human

monoclonals are therefore not as effective as human antibodies in clearing infected or diseased cells. And so, for a therapeutic antibody to be effective and to remain in the circulation without raising an anti-antibody response, it must be able to escape recognition by the recipient's immune system.

One solution to this problem is to form chimeric antibodies as described in Morrison et al (P.N.A.S., 1984, 81, 6851-6855); and Neuberger et al (Nature, 1985, 314, 268-270), where the variable region of a foreign species derived from a hybridoma as described above, is grafted to the constant region of human antibody. However, in this situation the variable region remains foreign to the recipient; thus, it may be recognised and may still pose a significant immunogenicity problem. The humanisation of an antibody, as described in Jones et al (Nature, 1986, 321 522-525); and Riechmann et al (Nature 1988, 332, 323-327), in which the CDRs of a foreign antibody species are grafted onto a human antibody framework, does alleviate many of these problems. However, the CDR-grafting of antibodies is a complicated process and the resultant antibody may require further modification to maintain its binding affinity. It is therefore clear that to avoid recognition by the human immune system, the optimum form of antibody is a human antibody. However, the process of obtaining human antibodies has its own problems.

Cell lines which secrete human antibodies against specific antigens are not readily available. However, cell lines have been produced from human lymphocytes but such lines have low stability, do not readily form hybrids which might be more stable, and usually produce low yields of antibodies when cultured in vitro. Furthermore it is possible that such cells may harbour foreign infectious nucleic acid, for example, from a virus, which poses problems of cross-infection. Lengthy purification and/or sterilisation procedures must be applied before the antibody produced therefrom is in an acceptable form for administration to humans.

European patent Publication No. 314161 discloses a process for the production of human immunoglobulin in a eukaryotic host cell. The host cell is transfected with operably linked first and second genes which code for human heavy chain variable and constant regions respectively. The host cell is also transfected with operably linked genes coding for variable and constant regions of a human light chain. This transfected cell is cultured and recombinant human immunoglobulins, having variable regions of the desired binding specificity, can be recovered from the cell culture. However, this process poses a number of problems.

1. A large quantity of lymphocytes is required for recovery of sufficient genomic DNA to carry out the process. The specification discloses (on pages 27-28), the removal of 1-2 x 10^8 lymphocytes from which 121μg of genomic DNA were retrieved.

2. All four alleles (two for the heavy chain and two for the light chain) are retrieved in the genomic DNA and require extensive sequencing and selection by cloning, expression and binding studies, to obtain the functional pairing of genes (one for heavy chain and one for the light chain) for further processing.

3. Poor expression levels of antibody are achieved by culturing the host cells transfected by the described process. The specification discloses yields of 6.7 - 34.5 μg/ml (page 38) which average out at 20 μg/ml, and using a construct including a cytomegalovirus (CMV) expression enhancer only 1μg/ml (page 40).

The present invention provides a new process which overcomes one or more of the problems associated with the prior art. The invention therefore provides a process for the production of a human antibody comprising:

(i) selecting a human lymphocyte-derived cell line that is capable of expressing a desired antibody;

(ii) isolating RNA from the cell line and separating mRNA from the other RNAs so isolated;
(iii) synthesising cDNA from the mRNA and inserting the cDNA into a cloning vector;
(iv) transforming a host cell with the vector containing the cDNA to obtain a library;
(v) screening the library for cDNA encoding the antibody;
(vi) inserting the cDNA encoding the antibody into an expression vector;
(vii) transfecting a host cell with the expression vector containing the cDNA; and
(viii) culturing the transfected host cell and isolating the desired antibody.

Reference to a human lymphocyte-derived cell line, means a cell line derived from a single human lymphocyte which will produce a single antibody. The cell line must be sufficiently stable to enable recovery of RNA and so is preferably stabilised or immortalised using conventional viral transformation and/or hybridoma technology (as described in Methods of Hybridoma Transformation, Bartal and Hirsaut (eds), Humana Press, Clifton, N.H. 1985). Such cell lines may be obtained from depositories such as the Amercian Type Culture Collection of Rockville MD, USA.

The cell line may be produced by removing lymphocytes, namely lymphoblastoid cells, B-lymphocytes, or plasmacytomas from the peripheral blood or from the spleen for example from an individual either known to have recovered from an infection by a pathogenic organism (eg virus or bacteria) wherein removal preferably takes place within two to three months post recovery, or from an individual who has received vaccination against a pathogenic organism, wherein removal preferably takes place within two to three months post immunisation. Lymphocytes may also be removed from an individual who is known to be suffering from cancer or an autoimmune disease. These

cells may then be stabilised by viral transformation and/or fusion as described below.

Viral transformation is preferably carried out using Epstein Barr Virus (EBV). Most peripheral blood B-lymphocytes have a receptor for EBV and when infected by the virus these cells are transformed with the accompanying expression of the EBV nuclear antigen (EBNA). However, only around 20% of cells are "immortalised" in vitro and these are in general only small non-activated B cells. Plasma cells (activated B-cells) lack the EBV receptor so resistance to EBV infection appears to increase with maturity reducing the effectivity of viral transformation by this route when the recovered lymphocytes are mature.

For viral transformation using EBV it is therefore preferable to use non-plasma cell peripheral blood lymphocytes. To establish a cell line, supernatant from a cell line producing the virus such as B95.8 (Miller et al 1972 Proc. Natl. Acad. Sci. USA 69 383-387) generally contains sufficient infectious virus particles. In practice pellets of up to 10^7 cells are suspended in approximately 1ml of the viral culture supernatant and incubated at 37°C for about 1 hour. This allows attachment of the virus to specific receptors on B-cells and cell penetration. It is preferable to agitate the container gently to prevent sedimentation. The cells so infected can then be cultured and the genes for the desired antibody can be cloned.

An alternative or an additional step to viral transformation is to fuse to myeloma cells to provide stabilisation (Crawford, D.H. 1985 Human Hybridomas and Monoclonal Antibodies Ed. E.G. Engelman, S.K.H. Foung, J. Larrick and A. Raubitschek pp 37-50 or Roder J.C. et al The Epstein-Barr virus-hybridoma technique ibid pp55-67). The myeloma is optionally a heterohybridoma preferably of mouse/human origin. A suitable heterohybridoma can be generated for example from an antibody secreting cell-line such as HTO1. Suitable cells can be selected on

the basis of their sensitivity to hypoxanthine aminopterin and thymidine by subjecting them to sequential passage through medium containing 8-azaguanine as they are aminopterin sensitive.

In order to use such a heterohybridoma, the genes encoding endogenous human heavy and light chains must be deleted, otherwise the final cell line will be capable of producing more than one antibody and will not contain heavy and light chains for the desired antibody alone. This can be achieved by subjecting the cells to a 90% lethal dose of ultra-violet irradiation and selecting for suitable colonies by cytoplasmic staining with anti-human Ig and chromosome number namely polyploid with between 60-140 mouse chromosomes.

It is also advantageous to select vigorously growing cells. This may be achieved by passaging through the peritoneal cavity of a 2,6,10,14-tetramethylpentadecane (or pristane) primed mouse.

Final selection for growth, karyotype and fusability is then carried out, karyotype being the most important. The ideal heterohybridoma contains mainly mouse chromosomes.

Selection of a human lymphocyte cell line may be carried out by screening for the production of antibody which has affinity to the desired antigen, and antibody functionality.

Testing for affinity can be achieved by immunoassay techniques for example radioimmunoassay or Enzyme Linked Immunosorbent Assay (ELISA). Immunoassay techniques such as these use the specific interaction of antibody with antigen to provide information about antigenic specificity. Radioimmunoassays assess antibody level either by determining the capacity of antibody to complex with radioactive antigen or by measuring the amount of antibody binding to an insoluble antigen preparation. The ELISA technique involves conjugating enzymes to antigens or antibodies. The enzymes are usually selected on the basis of simple kinetics and can be measured by a coloured reaction

product for example by spectrophotometry. Preferred enzymes include alkaline phosphatase, β-D-galactosidase and horseradish peroxidase. ELISA can be employed as a primary binding or a competitive binding assay. For example, lymphocytes isolated from an individual infected by a virus can be selected by culturing supernatant medium from one lymphocyte cell line in the presence of a suitably labelled viral antigen possibly in the form of whole or empty viral particles. The antibody/antigen complexes can then be identified as described above and the respective lymphocyte cell line selected for further studies.

The test for functionality of the antibody in the case of an infectious agent may involve competition studies for neutralisation for example viral neutralisation. Neutralisation studies can be carried out using Radioimmunofocussing Assay (RIFA), in which a fixed concentrate of purified antibody is cultured with equal volume of 10 fold dilutions of virus and then assayed for virus titre. An alternative test can be to use complement and test for cell lysis.

It is possible to obtain cells secreting human IgG, IgG1, IgG2 or IgG3 IgM, IgA, IgD or IgE. These can be selected by Ouchtolony agar double diffusion or ELISA.

Following selection of a lymphocyte cell line expressing a functional antibody with the desired specificity, the RNA can be isolated by standard techniques such as the method of Chomczynski and Sacchi (1987, Anal. Biochem. 162, 156-159). It is preferable to use between 1-3 x 10^4 and 1-3 x 10^7 lymphocyte cells to obtain this, but most preferably in the range 1-3 x 10^5 and 1-3 x 10^6 cells. In order to isolate the messenger RNA (mRNA) encoding the antibody, standard techniques are also employed as described in Molecular Cloning: A Laboratory Manual by Maniatis et al Cold Spring Harbor Laboratory Press.

Complementary DNA (cDNA) is synthesised from the mRNA by standard techniques as for example disclosed in the aforementioned Molecular Cloning Manual.

Identification and cloning of the cDNA which encodes the antibody heavy and light chain proteins can be achieved by cloning the cDNA into a replicable vector for example a plasmid, and transforming a host cell for example a prokaryote such as E.Coli. Eukaryotic cells may also be used. The resultant library can then be screened for antibody light and heavy chain cDNA.

Screening of this kind can be carried out using heavy and light chain DNA probes with detectable labels and a detection method as described in Gene Cloning by D.M. Glover (Published by Chapman and Hall Ltd London). These techniques can involve radiolabelling and detection by radiography methods or non-radioactive labels for example digoxigenin 11 dUTP and a detection kit for example the Nonradioactive DNA labelling and Detection kit available from Boehringer Mannheim. Clones can be selected and if desired the sequence of the antibody heavy and light chains can be determined.

It is also possible to introduce modifications into the antibody cDNAs at this stage prior to preparation of vectors for expression.

Once a suitable cell colony has been selected, the cDNA sequences for the light and heavy chain genes can be subcloned into vectors suitable for insertion into a host cell for expression. Construction of the expression vectors may be carried out in accordance with procedures known in the art (Molecular Cloning: A Laboratory Manual, Second Edition, Maniatis et al, Cold Spring Harbor).

The host cell must be capable of expressing the antibody in a functional form and therefore, should be a eukaryotic cell such as a mammalian cell e.g. myeloma or chinese hamster ovary (CHO) cells which are capable of carrying out post-translational modifications, in

particular, correct folding of the chains, and glycosylation, which can be essential for effective functionality of the constant region of an antibody. Such cells can be cultured in vitro quite successfully and are known to express functional antibody. Yeast or insect cells may also serve as host cells as they can also carry out desired post-translational modifications.

The heavy and light chain cDNA can be transfected in a single vector as described in WO87/04462, the method of which is incorporated herein by reference, or as a co-transfection. For expression in mammalian cells the vectors for co-transfection preferably contain independently selectable markers. Upon co-transfection into recipient cells, the resulting colonies may thus be selected for both markers. Colonies exhibiting the dual phenotype are generally capable of co-expressing both the light and heavy chains. The selectable markers may or may not be of a dominant nature. Examples of selectable markers include adenosine deaminase (Kaufman et al, P.N.A.S., 1989, 83 3136-40) asparagine synthetase (Cartier et al, Mol.Cell Biol., 1987, 7, 1623-28), E.coli trpB gene and Salmonella hisD gene (Hartman et al, P.N.A.S., 1988, 85, 8407-51), M2 mouse ribonucleotide reductase (Thelander et al, EMBO J, 1989, 8, 2475-79), human multidrug resistance gene (Kane et al, Gene, 1989, 84, 439-446), glutamine synthetase (Bebbington et al, DNA Cloning, Vol III, 1987, Ed. D.M. Glover, 163-188, IRL Press), xanthine guanine phosphoribosyl transferase (gpt) (Mulligan et al, Science, 1980, 209, 1422-27), hygromycin B (Santerre et al, Gene, 1984, 30, 147-156), neomycin gene (Southern et al, J. Mol. Appl.Genet., 1982, 1, 327 341), and dihydrofolate reductase (Subramani et al, Mol.Cell Biol., 1981, 1, 854-868).

A preferred selectable marker for use with one of the vectors is dhfr which is usually employed with a parental CHO cell line of the dhfr - phenotype (Urlaub et al, P.N.A.S., 1980, 77, 4216-4220). Successfully transfected CHO cells will possess the dhfr$^+$ phenotype and can readily be selected by culturing the colonies on media devoid of thymidine and

hypoxanthine and optionally containing methotrexate (MTX). A preferred selectable marker for use with the other of the vectors is a dominant resistance marker, such as neomycin (neo). CHO cells successfully transfected with this marker can readily be selected by culturing the colonies in media containing the antibiotic, Geneticin, an analogue of neomycin.

Particularly preferred for use in myeloma or CHO cells is the glutamine synthetase or GS system which is described in WO87/04462. Cells which have been successfully transfected with this marker can be selected by culturing colonies in media containing certain levels of methionine sulphoximine (Msx) as described in PCT published application number WO87/04462.

A selectable marker preferably also provides the basis upon which the genes encoding the light and heavy chains may be amplified. In transfection of a cell line, the vector DNAs are often integrated into the chromosome of the cell at the same locus. Thus, the use of a selectable marker as the basis for amplification normally results in a parallel increase in the copy number of both genes. One selectable marker for use in this way is dhfr which enables the desired amplification selection through the use of increasing concentrations of MTX. Also preferred is the GS marker which can be used to enable amplification selection by employing increasing concentration of Msx.

The selectable markers are of course under the control of regulatory elements of DNA so as to provide for their expression. The regulatory elements are preferably of a viral source, such as from DNA tumour viruses. Particularly preferred are the use of an SV40 or adenovirus major late promoter. It is particularly advantageous in this regard to remove the enhancer element from the promoter thus effectively "crippling" it. This modification allows for increased levels of gene amplification at each concentration of methotrexate selection than would otherwise occur if a strong promoter was used. In the case of the use of GS as a selectable marker, an example of a suitable

promoter is the mouse metallothionein promoter or preferably the human cytomegalovirus (hCMV)-MIE promoter described in PCT patent publication number WO89/01036.

The light and heavy genes are also under the control of regulatory elements of DNA so as to provide for their expression. The use of the same regulatory elements for both chains is preferred so that their expression is substantially balanced. The regulatory elements may be of viral origin and examples include those mentioned above in conjunction with the expression of dhfr or GS as a selectable marker. Another example is the use of the β-actin promoter and cognate β-actin polyadenylation signal.

One or both of the vectors may also contain an SV40 origin of replication to allow for the vector constructs to be checked by rapid transient assay for example in COS cells.

The invention therefore includes a vector suitable for transfection of a host cell comprising cDNA encoding human antibody heavy and light chains.

Co-transfection of the cell line with the expression vectors may be carried out simply by using equimolar quantities of both vectors and standard transfection procedures, such as calcium phosphate precipitation or lipofectin. Selection of the desired co-transfected cell line may be carried out in accordance with standard procedures known for the particular selectable markers.

The invention also includes a eukaryotic cell line transfected with cDNA for the expression of human antibody heavy and light chains.

The invention further includes a process for the expression of cDNA encoding human antibody heavy and light chains comprising transfecting a eukaryotic host cell with a vector or vectors containing said cDNA.

Culture of the cell line may be carried out in serum-containing or preferably serum-free media. Where the cell line is a CHO dhfr$^+$ transformant, the medium preferably lacks hypoxanthine and thymidine and optionally contains MTX. Expression of both chains in substantially equimolar proportions enables optimum yields of functional antibody to be obtained. The two chains assemble within the cell and are then secreted into the culture media as functional antibody. The resulting recombinant antibody may be purified and formulated in accordance with standard procedures.

The invention further comprises a human antibody produced by:

i) selecting a human lymphocyte derived cell line that is capable of expressing a desired antibody;
ii) isolating RNA from the cell-line and separating mRNA from the other RNA so isolated;
iii) synthesising cDNA from the mRNA and inserting the cDNA into a cloning vector;
iv) transforming a host cell with the vector containing the cDNA - to obtain a library;
v) screening the library for cDNA encoding the antibody;
vi) inserting the cDNA encoding the antibody into an expression vector;
vii) transfecting a host cell with the expression vector containing the cDNA; and
viii) culturing the transfected host cell and isolating the desired antibody.

The use of eukaryotic cell lines transfected with cDNA can be expected to yield greater than 50μg/ml of antibody preferably up to or more than 250μg/ml. -

A further aspect of the invention comprises a human antibody produced by the process of culturing a eukaryotic host cell line capable of expressing cDNA encoding human antibody heavy and light chains.

The resultant antibody can be used as a therapy, according to its specificity. The example provided hereinafter is an anti-hepatitis A antibody for use in the treatment of hepatitis A infections. Antibodies can be obtained which target other viruses such as herpes viruses : herpes simplex virus, cytomeglovirus, Epstein Barr virus, varicella zoster virus; or other pathogenic organisms. Cancerous cells are also possible targets for human antibodies. Optionally they can be used as targetting moieties which deliver chemical or biological compounds. These are incorporated into the cell by endocytosis where they are toxic or are metabolised to form a toxic agent, killing the cell. Also included are other anti-self antigens in autoimmune disease.

DESCRIPTION OF FIGURES

Figure 1. Nucleotide and deduced amino acid sequences of Antibody D heavy chain. The complete sequence of the pH210H2 insert is shown. The signal peptide and CDR sequences are underlined, and the predicted polyadenylation signal overlined. Amino acids are numbered according to Kabat et al. (1987).

Figure 2. Nucleotide and deduced amino acid sequences of Antibody D light chain. The complete sequence of the pH210H2 insert is shown. The signal peptide and CDR sequences are underlined, and two predicted polyadenylation signals overlined. Amino acids are numbered according to Kabat et al. (1987).

Examples

Production of human/mouse chimaeric cells for hybridisation

Production of cell-line HTO1

1. 50 ml blood taken from a healthy human donor, 7 days after booster immunisation with tetanus toxoid, and mixed with

preservative-free heparin as anti-coagulant. Mononuclear cells separated on Ficoll/Hypaque (Boyum A. 1986 Scand.J.Clin.Invest. 21,77-89), washed in Hanks buffered saline and fused with a mouse myeloma cell line by conventional techniques as follows:

2. NS-O mouse myeloma cells (Galfre G. and Milstein C. (1982) Immunology 45, 125-128) were harvested from a log-phase culture and washed in Hanks saline. Mononuclear cells ($4.7x10^7$) and NS-O cells ($6x10^7$) were mixed and centrifuged in a 50 ml test tube. The pellet of cells was then resuspended in 1 ml 50% polyethylene glycol solution and mixed gently for 1 minute at room temperature. The fused cells were resuspended in RPMI medium with 10% foetal calf serum and dispensed drop-wise into 60 1 ml aliquots of this growth medium in 24 well plates.

3. 24 hours later 1 ml of medium containing hypoxanthine aminopterin and thymidine (HAT) and $1x10^6$ Balb/c mouse spleen cells was added to each well. The plate was incubated at 37°C in 5% CO_2.

4. 20 days after fusion the supernatants were screened by radio-immunoassay for human anti-tetanus toxoid antibodies. One well containing one small colony of cells (approximately 20) was identified. This colony was slow-growing, a characteristic associated with prolonged stability of antibody secretion due to enhanced retention of human chromosomes.

5. These cells were transferred to a fresh well and after three weeks were cloned by limiting dilution (LD). All the subclones tested were positive in the Radioimmunoassay (RIA).

6. One clone, PB47 1.A1.B9.E10, was named HT01 and cells frozen down. These cells synthesised human IgMK anti-tetanus toxoid antibody.

7. Karyotypic analysis showed that the cells contained a polypoid modal number of mouse chromosomes and many human chromosomes (see Figure) . These cells were selected as starting material for production of a polypoid fusion partner for the preparation of further hybridomas.

The cell line HT01, which secreted human IgM anti-tetanus antibody was used as starting material to produce a polyploid fusion partner. Cells sensitive to HAT were selected by subjecting the aminopterin resistant HT01 cells to sequential passage through medium containing 8-azaguanine, from 1μg-20μg/ml.

In order to stimulate loss of the human antibody heavy and light genes, a sample of cells was subjected to a 90% lethal dose of ultra-violet irradiation. Irradiated cells were cloned at limiting dilution and a number of colonies were selected on the basis of lack of cytoplasmic staining with anti-human Ig and nuclear size which correlates with chromosome number. One clone, HT01.A was selected after karyotypic analysis showed it to be polyploid with between 60-140 mouse chromosomes.

Selection of vigorously growing HT01.A cells was achieved by passaging a sample through the peritoneal cavity of a pristane-primed mouse (PRISTANE is 2,6,10,14 - tetramethylpentadecane from Aldrich). Those cells that survived, grew as single colonies on microtitre plates. These were cultured and assessed for growth, karyotype and fusibility. One, designated HT01.A.P1 was finally selected on the basis of modal numbers of 135 mouse and 3 human chromosomes. This cell line was used as a fusion partner with the peripheral blood lymphocytes from the hepatitis A virus seropositive donor.

Removal and stabilisation of antibody secreting cells

A blood sample (30mls) was obtained from a hepatitis A virus (HAV) sero positive donor, approximately four months after an infection with hepatitis A contracted from contaminated food in the UK.
Peripheral blood lymphocytes were separated on a lymphoprep gradient (Flow Labs), transformed with Epstein Barr Virus (EBV) and cultured for ten days in medium containing phaetohaemaglutinin and 10% foetal calf serum. They were then fused with appropriate human/mouse chimeric cells as described above, using PEG 1500, and cultured in the presence of HAT and 10^{-5}M ouabin in 2 ml wells. Supernatant media were screened by sandwich ELISA (REF) for anti-HAV activity, ten days post-fusion when distinct colonies were visiable microscopically. Individual colonies were picked from positive wells, and monoclonal cell lines established from these by cloning twice from single cells at limiting dilution. Of the original 42 2ml wells, seventeen were strongly positive in initial ELISA screens following extensive re-feeding, but secreting monoclonal lines were only successfully established from four of these. The others ceased secreting antibody at various states of isolation or cloning, including after double cloning, presumably due to the inherent chromosomal instability of heterohybrids.

Selection of hybridoma

ELISA Studies

The four antibodies (A, B, C & D) from the cell lines described above, were titrated in both Sandwich and Direct ELISA (REF) against full and natural empty HAV particles. The titres, expressed as the log 10 reciprocal dilutions producing 50% of the maximum absorbance plateau, are shown in Table 1a. These values, expressed as percentages of the titres of individual antibodies against native particles in the Sandwich test are given in Table 1b.

ELISA TITRES OF HUMAN ANTIBODIES AGAINST FULL AND NATURAL EMPTY HAV PARTICLES

Table 1a

Antibody I.D.	Sandwich		Direct	
	Fulls	Empties	Fulls	Empties
Antibody A	4.42	4.42	3.77	3.35
Antibody B	4.85	4.78	4.13	3.80
Antibody C	3.91	3.72	4.02	3.59
Antibody D	4.10	4.15	3.92	3.60

ELISA ACTIVITY OF ANTIBODIES EXPRESSED AS PERCENTAGE OF THE HOMOLOGOUS ANTIBODY TITRE AGAINST FULL PARTICLES IN THE SANDWICH TEST

Table 1b

Antibody I.D.	Sandwich		Direct	
	Fulls	Empties	Fulls	Empties
Antibody A	100	100	22	9
Antibody B	100	85	19	9
Antibody C	100	65	126	48
Antibody D	100	112	66	32

Competition Studies

The ability of the antibodies to inhibit both each other and murine antibodies from binding to the virus was carried out using solid phase radioimmunoassay (RIA) and ELISA techniques, which only differ at the final stage. The results, expressed in Table 2 as the maximum competition (%) obtained between antibody pairs, show that;

I) Antibodies A and B are indistinguishable and are similar to the K24F2 murine antibody (MacGregor A. et al 1983, J.Clin.Microb. 18 page 1237).

II) Antibody D is closer in nature to murine antibody B5B3, (Stapleton J.T. and Lemmon S.M. 1987 J. Virol. 61 p491) and only interferes with Antibody A to a maximum of approximately 30% in reciprocal tests.

III) Antibody C appears to be functionally intermediate between Antibody A and D.

IV) Both Antibody A and Antibody D were individually able to inhibit the binding of human HAV polyclonal sera (Lemmon S.M. et al 1983 J.Clinical.Microb. 17 page 834 namely-Foxwell and Chulay) very efficiently.

The high competition values obtained with Antibodies A and B against B5B3 were obtained with 10-fold concentrated antibody, whereas the tissue culture supernates produced only 20% competition or less. In contrast, the same supernates required substantial dilution to obtain full competition curves against the K24F2 and K34C8 antibodies (MacGregor A.et al 1983, J.Clin.Microb. 18 page 1237) as did the Antibody D supernate against B5B3.

MAXIMUM COMPETITION (%) OF ANTIBODY BINDING TO THE 18F HAV STRAIN

Table 2

4th Ab (Detection) / 3rd Ab (Competitor)	A	D	K34C8	K24F2	B5B3	Human Polyclonal Antiserum (Chulay)
Antibody A	100	32	90	100	96[1]	94
Antibody B	100	33	78	100	>65[1]	
Antibody C	66	55	46	72	91	
Antibody D	24	100	29	69	99	92
Antibody A&D 1:1 mix						99
K34C8	100	32	100			
K24F2	100	70		100		
B5B3	69	100			100	
Human polyclonal antiserum (NF)						100

(1) Concentrated antibody required for maximum competition

RIFA STUDIES

All antibodies detected by the initial screening ELISA were also positive in Radioimmunofocussing Assay against the 18f virus. (Daemer R.J. et al (1981). Infec & Immunol. 32 page 388; and Stapleton J.T. and Lemmon S.M. (1987) J.Virol Vol 61 p492; and Ping L.A. et al 85 p821). The reductions in virus RIFA titres, obtained from reacting equal volumes of a fixed concentration (1mg/ml) of affinity purified antibody with 10-fold dilutions of the 18f and 43c virus strains (43c strain was derived from 18f strain by passaging under pressure from a murine monoclonal), are summarised in Table 3. These demonstrate that mutant 43c is very poorly neutralized by either antibody but shows significant, albeit reduced neutralization with polyclonal Foxwell serum.

Although both antibodies appear to 'neutralize' 18f virus far less efficiently than polyclonal serum, this is largely due to a fairly constant number of residual plaques surviving at each virus dilution and consequently distorting the Spearman-Karber calculation of titre. Reaction with polyclonal serum, however, eliminated all plaque formation. Similar reaction slopes of the antibodies to polyclonal sera in competition studies, and their overall ELISA reactivity, give no indication that these represent low affinity antibodies.

REDUCTION IN TITRE LOG_{10} PLAQUE FORMING UNITS/ML OF HAV ISOLATES REACTED WITH SPECIFIC ANTIBODY

HAV strain	Antibody A (1mg/ml)	Antibody D (1mg/ml)	Polyclonal human serum, (1/10)
18f	3.15	2.72	>4.56
43c	0.68	0.78	1.93

Cloning and sequencing of the anti-hepatitis A virus monoclonal Antibody D heavy and light chains

Total RNA was isolated from $2.5x10^7$ Antibody D expressing cells following the method of Chomczynski and Sacchi (1987 Anal. Biochem. 162, 156-159), using 1ml of extraction solution per $1x10^7$ cells. The resulting RNA pellet was redissolved in 50µl diethyl pyrocarbonate (DEPC)-treated distilled water, and spectrophotometrically determined to be at a concentration of 4.4µg/µl. Dynabeads Oligo $(dT)_{25}$ (Dynal) was used to extract mRNA from 75µg total RNA employing the manufacturer's protocol.

cDNA was synthesised from the isolated mRNA and cloned into the plasmid pSPORT-1 using the SUPERSCRIPT Plasmid System for cDNA Synthesis and Plasmid Cloning kit (Bethesda Research Labs - BRL) following the method recommended by the manufacturer. Escherichia coli MAX EFFICIENCY DH5 Competent Cells (BRL) were transformed with the resulting cDNA/pSPORT-1 ligation. Approximately 4500 colonies were lifted onto Hybond-N nylon filters (Amersham) and lysed, denatured and fixed following the method of Buluwela et al. (Nucleic Acids Res, 1989 17 452). The filters were treated with proteinase K (50μg/ml in 0.2xSSC, 0.1% SDS at 55°C for 30 min), and then excess debris removed with a tissue.

A human IgG1 antibody cDNA insert (rat CAMPATH-1 antibody heavy chain CDRs reshaped on human NEW IgG1 antibody heavy chain; Riechmann et al., 1988 Nature 382 323-327) was labelled with digoxigenin-11-dUTP using the Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim) and employed to screen filters, possessing approximately 500 lifted colonies, for Antibody D heavy chain following the manufacturer's protocol. Two potential positive colonies were detected and selected for further analysis. Plasmid DNA was prepared using the method of Del Sal et al. (1988 Nucleic Acids Res 16 9878) and both of the potential positive clones were found to contain inserts of the expected size for human immunoglobulin heavy chain cDNA. A clone, pH21OH2, was selected, and sequenced in both directions by plasmid priming following the dideoxy chain termination method (Sanger et al., Proc. Natl. Acad Sci USA, 1977 74 5463-5467), according to the Sequenase kit (United States Biochemicals - USB) protocol. The sequence of the variable region is shown in Figure 1.

A human lambda cDNA was labelled with digoxigenin-11-dUTP using the Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim) and employed to screen filters, possessing approximately 4000 lifted colonies, for antibody D light chain following the manufacturer's protocol. Twenty potential positive colonies were detected and 10 selected for further analysis. Plasmid DNA was prepared using the

method of Del Sal et al. (1988) and 8 contained inserts of the expected size for human antibody light chain cDNA. A clone, pH210L2, was selected, and sequenced in both directions by plasmid priming following the dideoxy chain termination method (Sanger et al., 1977), according to the Sequenase kit (USB) protocol. The sequence of the variable region is shown in Figure 2.

ASSEMBLY OF EXPRESSION CONSTRUCTS

The expression vector pRDN1 was adapted from the pLD9 plasmid (described in Page, M. and Sydenham M.A. (1991) Biotechnology 9 64-68) as follows. The HindIII site used to insert the SV_{40} origin of replication and the other HindIII site 5' to the DHFR coding sequence were destroyed by HindIII digestion, filled-in with klenow fragment of DNA polymerase, and re-ligated. A clone lacking both restriction sites was digested with $EcoR_1$, filled-in with klenow enzyme and re-ligated. The resulting plasmid, lacking all internal HindIII and $EcoR_1$, sites, was used to insert the human β Actin expression cassette downstream of the DHFR transcripiton unit. This plasmid, has a functional SV40 origin of replication and pRDN1 has unique HindIII and $EcoR_1$, restriction sites downstream of the β Actin promoter. The adapted pRDN1 vector was digested with EcoRI, blunted with Klenow enzyme and dephosphorylated using calf intestinal phosphatase. The Antibody D heavy and light chain inserts were cut out of their respective clones, pH210H2 and pH210L2, using HinddI11 and $EcoR_1$, and blunt ended with Klenow enzyme. The blunt ended inserts were ligated into pRDN1 and used to transform Escherichia coli MAX Efficiency DH5 Competent Cells (Bethesda Research Labs BRL). Small-scale plasmid preparations (Del Sal, G. et al. (1988) Nucleic Acids Res. 16 9878) were carried out on a number of the resulting colonies and the inserts orientated using appropriate restriction digests. Plasmid DNA was prepared from one heavy and one light chain clone (pRDHH9 and pRDHL27 respectively) using QIAGEN (trademark) columns (Hybaid) following the manufacturer's protocol.

Transfection of COS cells

2×10^5 COS cells were plated in D-MEM (plus serum) in each well of a 12-well tissue culture dish. After 24 hours, the cell monolayers were rinsed twice with serum-free medium followed by 0.5 ml of serum-free medium containing 1 μg of each DNA construct (pRDHH9 and pRDHL27) and 5 μg TRANSFECTAM (Northumbria Biologicals Limited), as recommended by the manufacturer. After further incubation for 6 hours, the transfection medium was aspirated and replaced with 1 ml D-MEM (plus serum). After 48-72 hours, the medium was removed and assayed for human antibody.

ELISA Assay for Human Antibody

The medium from the COS cell transfection was assayed for the presence of human antibody. In the absence of light chain synthesis, heavy chains are not secreted from a cell and are degraded internally (Hendershot L. et al. Immunol. Today 8 111-114). Antibody can thus be assayed by detection of heavy chain in the culture medium. Microtitre plates were coated with anti-human IgG and incubated with the culture medium. Antibody was detected by visualisation with an anti-human gamma chain specific peroxidase conjugate.